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SJA6017, a newly synthesized peptide aldehyde inhibitor of calpain: amelioration of cataract in cultured rat lenses

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Abstract

The purposes of this experiment were to: (1), characterize the peptide aldehyde SJA6017, *N*-(4-fluorophenylsulfonyl)-L-valyl-L-leucinal, a newly synthesized inhibitor of calpain, and (2) test the effect of SJA6017 in preventing calcium ionophore-induced cataract in cultured rat lenses. In vitro, SJA6017 strongly inhibited purified *m*-calpain from porcine kidney. Casein zymography confirmed that SJA6017 reversibly bound to the active site of *m*-calpain. SJA6017 was also confirmed to be a cell-permeable inhibitor in Molt-4 cells. In cultured lenses, SJA6017 reduced nuclear opacity and proteolysis of crystallins and α-spectrin caused by calcium ionophore A23187. These results suggested that SJA6017 is a reversible and cell-permeable calpain inhibitor which may possess great efficacy against calcium-induced models of cataract. © 1997 Elsevier Science B.V.

Keywords: SJA6017; Calpain; Calcium ionophore A23187; Cataract; Proteolysis

1. Introduction

Calpains (EC 3.4.22.17) are non-lysosomal, cysteine proteases with a neutral pH optimum, and they are activated by calcium [1]. Activated calpain is thought to be involved in cataract formation in rodent lenses for the following reasons: (1) Increased lens calcium is found in many types of rodent cataracts [2–4]; (2) the cleavage sites on the N-terminal extensions on β-crystallins in insoluble lens proteins in cataractous rodent lenses are very similar to those found on β-crystallin polypeptides incubated with purified *m*-calpain [5,6]; (3) inhibitors of cysteine proteases such as calpain decreased proteolysis and temporarily prevented the progression of cataract formation caused by various inducers in vitro and in vivo [7–9].

A number of calpain inhibitors are reported in the literature and these inhibitors were used in studies to determine the role of calpain in disorders including cataract [10]. The most widely used inhibitors are probably the peptide aldehydes such as leupeptin. The aldehyde end group binds the sulfhydryl group of the active site cysteine to trap the enzyme. This binding is Ca^{2+} dependent and reversible [10]. Unfor-

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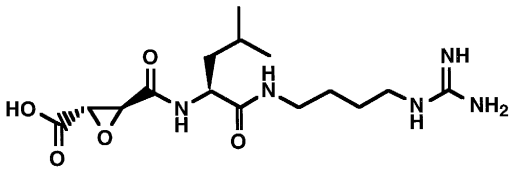
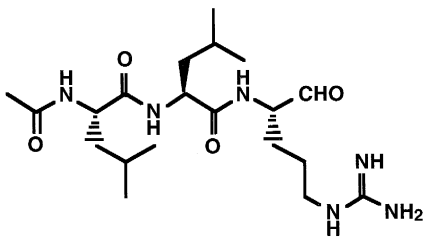
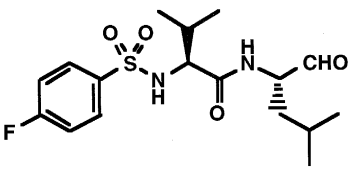
tunately, cell permeability of leupeptin is low because of the presence of the positively charged arginine residue. Other widely used inhibitors are the epoxysuccinyl peptides such as E64. This molecule covalently binds to a critical sulfhydryl group in the active site of calpain, and this binding is irreversible [10]. E64 prevents proteolysis of crystallins and temporarily slows formation of nuclear cataract in vitro and in vivo, although the membrane permeability of E64 is relatively poor [8]. However, the effect of E64 against an in vivo animal model was weaker than against in vitro lens culture system. We hypothesized that the amount of E64 penetrating into the lens were not high enough to prevent proteolysis of crystallins and subsequent opacity [7]. For more effective prevention of cataract, a new peptide aldehyde inhibitor SJA6017, *N*-(4-fluorophenylsulfonyl)-L-valyl-L-leucinal, offering stronger inhibition of calpain, or more effective cell penetration, was synthesized (Fig. 1). Thus, the purpose of the present experiment was to test the efficacy of SJA6017 in prevention of proteolysis of lens proteins and nuclear opacity in calcium ionophore-induced cataract in cultured lenses. We found that prevention of proteolysis and cataract by SJA6017 were related to their strong inhibition of calpain and extensive membrane permeability.



E-64



Leupeptin



SJA6017

Fig. 1. Structures of calpain inhibitors used in the present study.

2. Materials and methods

2.1. Synthesis of SJA6017

Treatment of L-valine with 4-fluorobenzenesulfonyl chloride gave *N*-(4-fluorophenylsulfonyl)-L-valine which was coupled with L-leucinol by active ester method to *N*-(4-fluorophenylsulfonyl)-L-valyl-L-leucinol. DMSO oxidation of *N*-(4-fluorophenylsulfonyl)-L-valyl-L-leucinol using sulfur trioxide-pyridine complex gave *N*-(4-fluorophenylsulfonyl)-L-valyl-L-leucinal (SJA6017). The compound synthesized was confirmed by NMR and elemental analysis.

2.2. Enzyme assay

Calpain enzyme activity assays were performed using FITC-labeled casein as substrate in a 30-min assay at 30°C [8]. Inhibition of calpain activity by cysteine protease inhibitors was measured in the presence of 0.4 U of *m*-calpain purified from porcine kidney (Nacalai Tesque, Japan).

2.3. Proteolysis of α-crystallin and autolysis of m-calpain

α-Crystallins were purified from rabbit lenses using gel filtration chromatography [11]. Experimental animals were handled in accordance with the Declaration of Helsinki and appropriate National Institutes of Health recommendations. Twelve units purified *m*-calpain/ml were incubated with 100 μg/ml of α-crystallin and 1.5 mM free calcium in buffer B containing 50 mM Tris (pH 7.4), 1 mM EGTA, 3 mM dithiothreitol at 30°C. The reaction was then stopped by the addition of excess EDTA. To test for inhibition of proteolysis of α-crystallin, 100 μM SJA6017, 500 μM E64 (Peptide Institute, Osaka Japan) or 500 μM leupeptin (Peptide Institute, Osaka Japan) were used. SDS–PAGE of incubation mixtures was performed on discontinuous, 12% gels [12].

2.4. Casein zymography

Casein zymography was performed using the method of Racer et al. [13]. Note that all zymograms were soaked in the calcium buffer after electrophoresis so that calpain activity could be subsequently visualized in the gels as lysis of casein. Pre-incubated calpain samples were produced by incubating 12 units purified *m*-calpain/ml with 1.5 mM calcium, or without calcium, in buffer B at 30°C. One hundred μM SJA6017, 500 μM E64 or 500 μM leupeptin were added to test for inhibition of *m*-calpain autolysis. Calpain activity in the casein gels was visualized by staining with Coomassie brilliant blue R-250, yielding a white band indicating calpain proteolysis. Experiments were also performed where calpain was first incubated with calcium (Fig. 4, lower), then after subsequent zymography, *no* band of lysis was observed because the calpain had degraded itself (autolysis).

2.5. Molt-4 cell culture

To test the ability of inhibitors to permeate cell membranes and to inhibit cellular calpain activity, human lymphoid cell line MOLT-4 (ATCC No. CRL 1582) was used. Degradation of α-spectrin by *m*-calpain in this cell line following treatment with calcium ionophore A23187 has been well established [14]. MOLT-4 was subcultured as recommended by American Type Culture Collection. Two million MOLT-4 cells/ml were preincubated for 1 h with inhibitors. To activate endogenous calpain, 5 μM of calcium ionophore A23187 were added, and the cells were further incubated for 1 h at 37°C. Cells were then collected by centrifugation and total cellular protein for measurement of α-spectrin was extracted as described [15]. Protein content was measured by BCA assay (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as a standard. Tricine SDS–PAGE of total proteins were performed on discontinuous, 10% gels (Tefco, Japan) [16]. Immunoblots for α-spectrin were performed by electrotransferring proteins from SDS–PAGE gels onto PVDF membrane (Millipore) using the method of Towbin et al. [17]. A mouse anti-α-spectrin (nonerythroid) monoclonal antibody (Chemicon International Inc., CA) was used at 1:500 dilution, and immunoreactivity was visualized with alkaline phosphatase conjugated to anti-mouse IgG secondary antibody and BCIP/NBT (Bio-Rad). The staining intensity of the immunoblots of intact α-spectrin antigen were determine by densitometric image analysis, since the standard curves gave proportional increases in α-spectrin staining as the amount of total protein from MOLT-4 was increased (data not shown).

Statistical analysis of data was performed by Dunnett multiple comparison.

2.6. Lens culture

For lens culture studies, lenses from 5-week-old Sprague-Dawley rats were cultured at 37°C under 5%

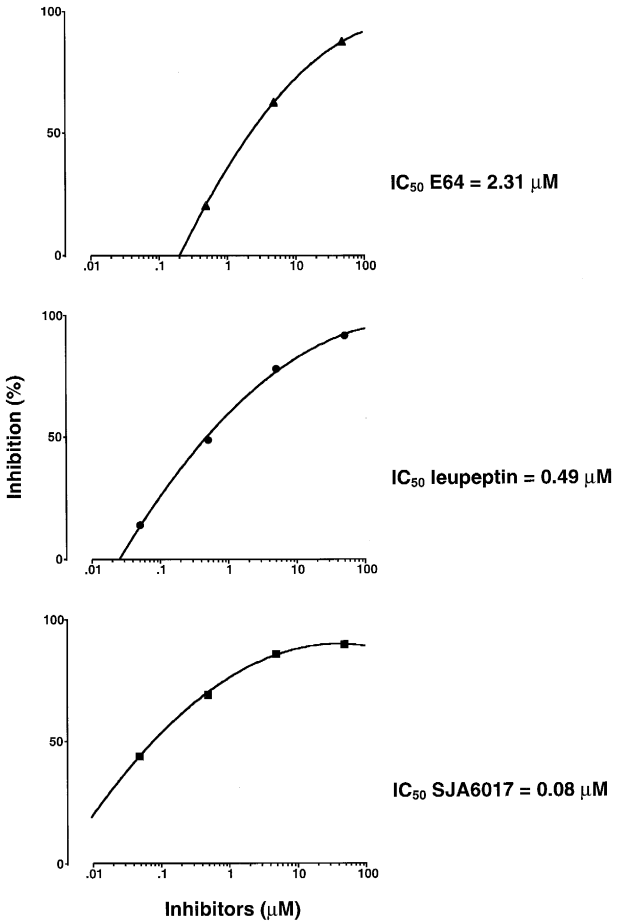


Fig. 2. Inhibition of purified *m*-calpain by E64 (top), leupeptin (middle) or SJA6017 (bottom) in vitro. Inhibitors were tested against 0.4 U *m*-calpain in a caseinolytic assay. This is a representative experiment repeated two times.

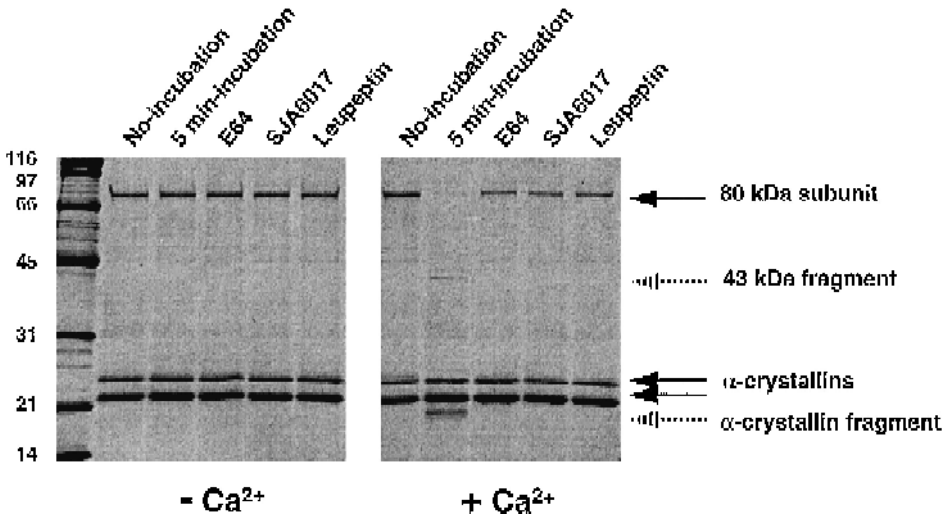


Fig. 3. Representative SDS–PAGE measuring proteolysis of α-crystallins and autolysis of *m*-calpain with (right gel) and without (left gel) Ca^{2+} in incubation mixtures. Lanes marked 'No-incubation' are *m*-calpain and α-crystallins mixed but not incubated. Lanes marked '5 min-incubation' are *m*-calpain and α-crystallins incubated for 5 min. Next lanes are incubated *m*-calpain and α-crystallins plus inhibitors (lanes marked 'E64', 'SJA6017' or 'leupeptin') for 5 min. Molecular weight standards are indicated in kDa on the left. Note decreases in bands at approximately 80 kDa (*m*-calpain 80-kDa subunit, solid arrow) and 22 and/or 24 kDa (α-crystallins, solid arrows), and new bands at 43 kDa (degraded calpain, stippled arrow) and 19 kDa (α-crystallin fragment, stippled arrow); these changes were prevented by E64, SJA6017 and leupeptin.

CO_2 in 4 ml Eagle's minimum essential medium (MEM, Gibco) with 10% fetal bovine serum (Gibco) (Normal group). This culture medium was used since lens transparency could be maintained for at least 15 days [18]. Ten μM calcium ionophore A23187 was present on day 1 only (A23187 group), and 100 μM

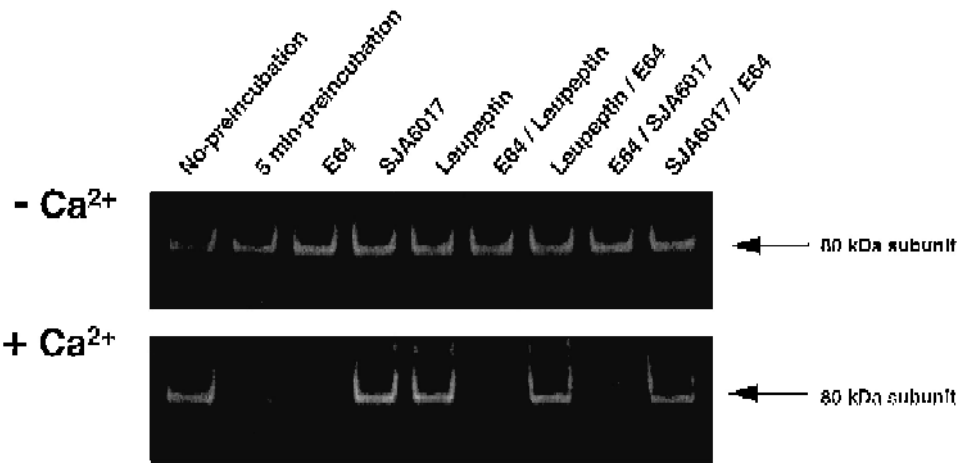


Fig. 4. Representative casein zymograms for *m*-calpain enzyme activity. Note that all zymograms were soaked in Ca^{2+} buffer after electrophoresis to show calpain activity remaining in the gel. This figure also shows results of an experiment were calpain solutions were pre-incubated with or without Ca^{2+} to test for autodegradation. White areas show caseinolysis at the active 80-kDa subunit of *m*-calpain. Gels show caseinolytic activity of *m*-calpain after pre-incubation with (lower) and without (upper) Ca^{2+}. Lanes marked 'No-preincubation' are *m*-calpain without pre-incubation. Lanes marked '5 min-preincubation' are *m*-calpain pre-incubated for 5 min. Next lanes are pre-incubated *m*-calpain plus inhibitors (lanes marked 'E64', 'SJA6017' or 'Leupeptin') for 5 min. Last 4 lanes are pre-incubated *m*-calpain plus E64 (lanes marked 'E64/Leupeptin'), leupeptin (lanes marked 'Leupeptin/E64'), E64 (lanes marked 'E64/SJA6017') or SJA6017 (lanes marked 'SJA6017/E64') for 5 min followed by leupeptin, E64, SJA6017 or E64 for an additional 5 min.

SJA6017, E64 or leupeptin were present continuously (A + SJA6017, E64 or leupeptin group, respectively). After 5 days of culture, lenses were photographed under a dissecting microscope, and density of lens opacity was quantitated using computerized image analysis (Image 1.31 software, Twilight clone BBC, Silver Springs, MD). After homogenization, soluble and insoluble proteins were obtained by centrifugation [8], and protein contents were measured by BCA assay. SDS–PAGE of proteins were performed on discontinuous, 12% gels [12]. The staining intensity of gels was measured using densitometric image analysis, and breakdown of α-crystallin in the soluble proteins was used as a marker for proteolysis because α-crystallin is lost in rat lenses cultured with cataractgenic agents [8]. Immunoblots for α-spectrin of soluble and insoluble proteins were performed using the same protocol as MOLT-4 cells. For analysis of crystallins and α-spectrin, whole lenses were used although the opacities of lenses were mainly in the nucleus. Crystallins and α-spectrin are distributed in cortex and nucleus of the lens [19], and proteolysis of crystallins was found in both regions [20].

3. Results and discussion

The three compounds tested inhibited purified *m*-calpain in a concentration-dependent manner (Fig. 2). SJA6017 was the most effective with an IC_{50} values of 0. 08 μM.

In vitro incubation of α-crystallins with *m*-calpain and Ca^{2+} caused a decrease in intact α-crystallin polypeptides along with the accumulation of new, lower molecular weight fragments at approximately 19 kDa (Fig. 3, right). The 80-kDa subunit of *m*-calpain also decreased as incubation time with Ca^{2+}, and a 43-kDa fragment of *m*-calpain appeared (Fig. 3, right). In contrast, when α-crystallins and *m*-calpain were incubated without Ca^{2+}, no proteolysis was observed (Fig. 3, left). SJA6017, E64, and leupeptin all inhibited proteolysis of α-crystallins and the decrease in 80-kDa *m*-calpain (Fig. 3, right).

Casein zymograms showed that the band at 80 kDa representing *m*-calpain enzyme activity decreased due to autolysis during pre-incubation for 5 min with Ca^{2+} (Fig. 4, lower). The band of *m*-calpain activity did not decrease when the pre-incubation solution did not contain Ca^{2+} (Fig. 4, upper). Note that no decrease in *m*-calpain activity occurred because the original incubation before zymography contained no Ca^{2+}, and calpain activation and subsequent autodegradation did not occur. All zymograms were then soaked in buffer with Ca^{2+} to reveal remaining calpain enzyme activity. Binding of calpain inhibitors to *m*-calpain was assessed by visualizing white bands at 80 kDa on casein gels as a maker of remaining calpain activity after pre-incubation. Appearance of white bands on casein gels indicated that inhibitors were reversible, because the inhibitors were removed

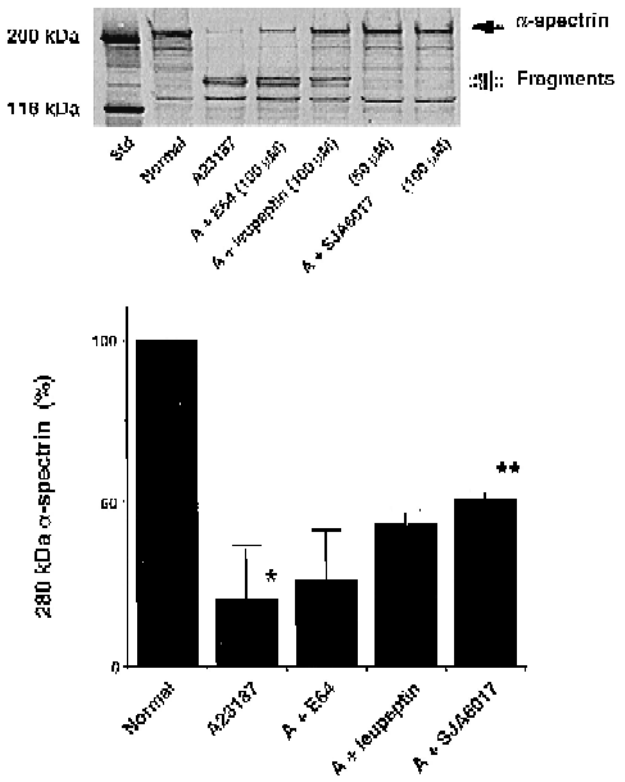


Fig. 5. Representative immunoblot (upper) and densitometric image analysis (lower) for α-spectrin in total protein (25 μg/lane) from cultured MOLT-4 cells showing proteolysis of α-spectrin after A23187 treatment and inhibition by calpain inhibitors. Cells were cultured without (Normal) or with A23187; or with A23187 plus E64 (A + E64), leupeptin (A + leupeptin) or SJA6017 (A + SJA6017). Molecular weight standards are indicated in kDa on the left. Note decreases in bands at approximately 280 kDa for α-spectrin (solid arrow), and new bands at 145 and 150 kDa (stippled arrows); these changes were partially prevented by SJA6017 and leupeptin, although SJA6017 was more effective than leupeptin. Data are means ± S.D. ($n = 5$). * $P < 0.01$ relative to Normal and ** $P < 0.05$ relative to A23187.

from *m*-calpain by electrophoresis. On the other hand, lack of white bands on casein gels indicated that inhibitors were irreversible; they were not removed from *m*-calpain after electrophoresis because of covalent binding between calpain and the irreversible inhibitor. When pre-incubations with Ca^{2+} included the irreversible inhibitor E64, *m*-calpain enzyme activity was inhibited. The band of *m*-calpain activity was not inhibited by E64 when Ca^{2+} was absent from the pre-incubation solution. This result showed that E64 irreversibly bound to *m*-calpain only when Ca^{2+} was present. Without Ca^{2+}, E64 could not bind to *m*-calpain in the initial incubation, and caseinolysis occurred during Ca^{2+} development of the zymogram once E64 was removed by electrophoresis. In contrast, *m*-calpain was reversibly inhibited by leupeptin. The band of *m*-calpain activity visualized in the zymogram was not decreased by pre-incubation with leupeptin and Ca^{2+}. Electrophoresis removed the reversible calpain inhibitor leupeptin, and caseinolysis could be observed in the zymogram. This indicated that, in contrast to E64, leupeptin was reversibly bound to *m*-calpain. When *m*-calpain was pre-incubated with leupeptin before *m*-calpain was further inactivated by E64, the band of *m*-calpain activity was not decreased. Leupeptin could preoccupy the active site and E64 no longer bound to active site of *m*-calpain. Results with SJA6017 were very similar to the reversible calpain inhibitor leupeptin. The band of *m*-calpain activity visualized in the zymogram was not decreased by pre-incubation with SJA6017 and Ca^{2+}. Thus, SJA6017 bound to the active site of calpain as E64. However, binding of SJA6017 was reversible in contrast to the irreversible inhibitor E64. Reversible inhibitors allow recovery of enzyme activity by dissociation, even though reversible inhibitors bind to non-target enzymes initially [21]. This may be produce fewer side-effects because none of calpain inhibitors reported in the literature are highly selective for calpain. That is, most calpain inhibitors cross react with other cysteine proteases such as cathepsins [22].

In cultured MOLT-4 cells, treatment with A23187 caused a decrease in intact α-spectrin at approxi-

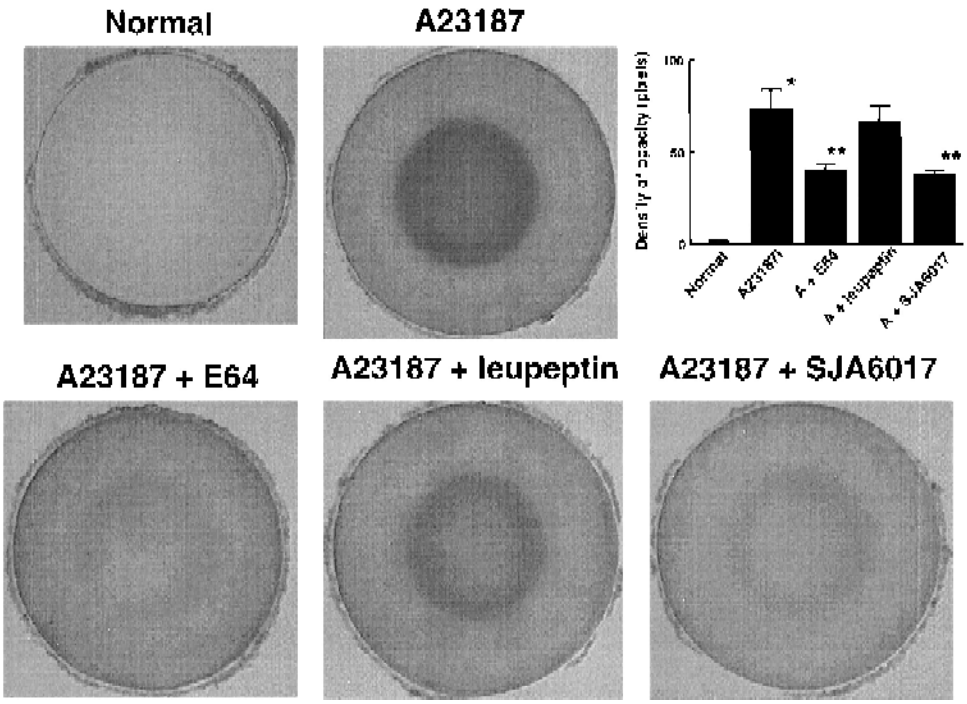


Fig. 6. Representative photomicroscopy of calcium ionophore-induced cataract and inhibition by calpain inhibitors on day 5 of culture. Darker areas are cataracts in these backlit lenses. Insert bar graph shows increased density of nuclear opacity in cultured lenses treated with calcium ionophore and reduction of opacity by calpain inhibitors. Data are means ± S.D. ($n = 5$). $*$ $P < 0.01$ relative to Normal and $**$ $P < 0.01$ relative to A23187 alone and A + leupeptin.

mately 280 kDa along with the accumulation of new, lower molecular weight fragments at 145 and 150 kDa (Fig. 5, upper). These two fragments are produced sequentially [14]. Note that SJA6017 and leupeptin partially prevented proteolysis of α-spectrin and inhibited production of the 145-kDa fragment. E64 did not inhibit proteolysis of α-spectrin and production of the 145-kDa fragment. Measurement of 280-kDa intact α-spectrin bands by image analysis confirmed that SJA6017 was more effective than leupeptin (Fig. 5, lower). This suggested that SJA6017 was more cell permeable than leupeptin or E64, because SJA6017 was able to prevent breakdown of α-spectrin more effectively. However, calpain inhibitors used in this study did not completely inhibit proteolysis of α-spectrin since a 150-kDa α-spectrin fragment appeared. One possible reason for this result is that the cleavage site of α-spectrin to produce the 150-kDa fragment is highly sensitive to protease [23]. The other possibility is that the ICE family of proteases also produced a 150-kDa α-spectrin fragment which was nearly identical to calpain-produced 150-kDa fragment in electrophoretic mobility, although both fragments have different N-termini [24].

Lenses cultured for 5 days in MEM remained clear and appeared similar to fresh lenses (Fig. 6). The toxic effect of SJA6017 on lens cultures was first assessed. The appearance of lenses cultured with SJA6017 alone was similar to fresh lenses (data not shown). Addition of 10 μM A23187 during the first day of culture caused a hazy opacity to appear in the peripheral region and a dense opacity in the central region of the lens by day 5 of culture. The effectiveness of the inhibitors in preventing these opacities was tested. Fig. 6 shows the typical appearance of A23187-treated lenses following culture with calpain inhibitors. SJA6017 and E64 were able to protect lenses against calcium ionophore-induced nuclear cataract. On the other hand, leupeptin did not reduce nuclear opacity. Measurement of the mean density of nuclear opacity in the lenses by image analysis confirmed the effect of inhibitors (Fig. 6, insert bar graph).

Analysis of the constituent polypeptides in the soluble fraction of normal lenses by SDS–PAGE revealed a characteristic predominance of polypeptides belonging to lens crystallins in the molecular weight range of 19–31 kDa (Fig. 7, upper, lane Normal). A23187-induced cataract exhibited proteolysis of crystallins (Fig. 7, upper, lane A23187). For example, the 31-kDa β- and 20-kDa α-crystallins were decreased, along with the accumulation of new polypeptide bands at approximately 27 kDa and 18

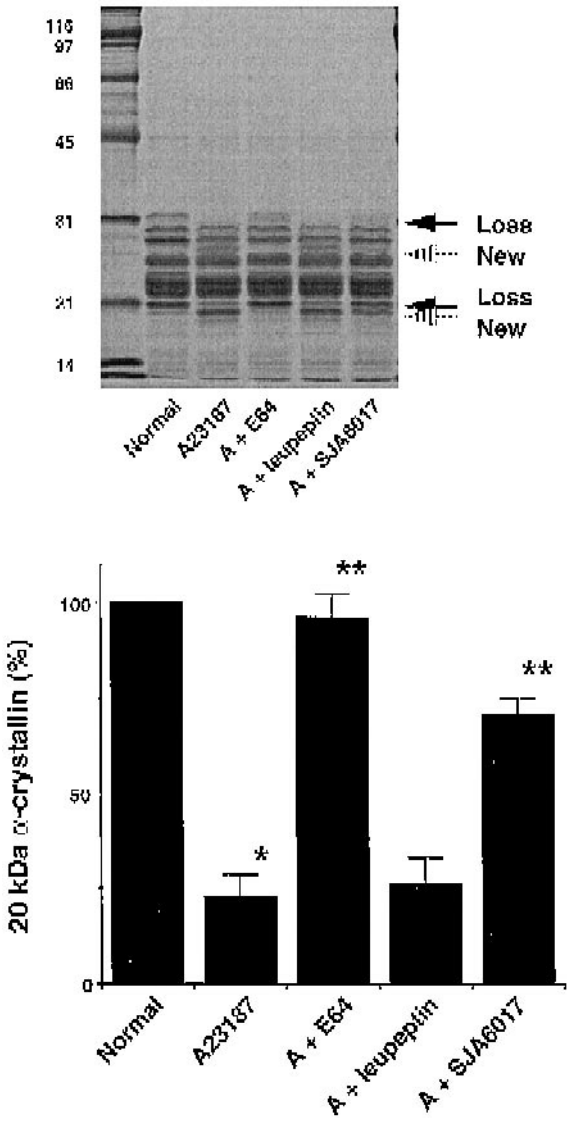


Fig. 7. Representative SDS–PAGE of soluble proteins (5 μg/lane) from lenses (upper) and densitometric image analysis for 20-kDa α-crystallin polypeptide (lower). Molecular weight standards are indicated as kDa on left. In cataractous lenses, note decrease of bands at approximately 31 and 20 kDa (solid arrows), and new bands at 27 and 18 kDa (stippled arrows); these changes were prevented by E64 or SJA6017. Data are means ± S.D. ($n = 5$). $*$ $P < 0.01$ relative to Normal, $**$ $P < 0.01$ relative to A23187 alone and A + leupeptin.

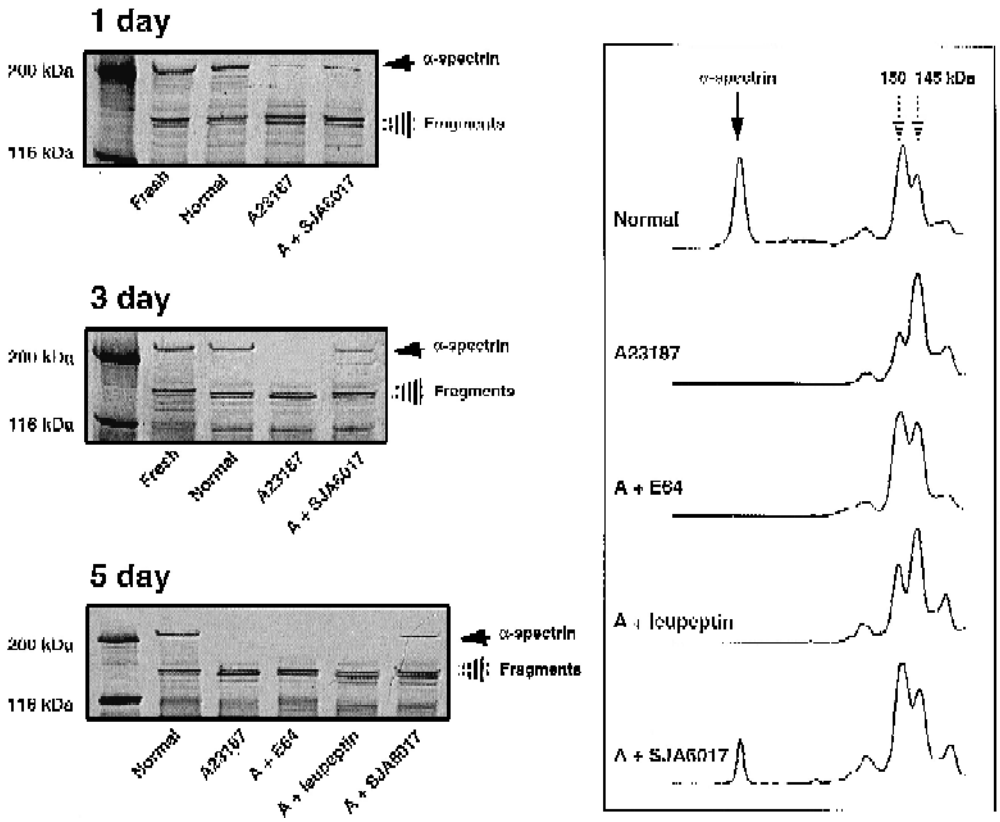


Fig. 8. Representative immunoblots for α-spectrin in the insoluble proteins (40 μg/lane) from lenses cultured for 1, 3 and 5 days (left) and densitometric scanning of immunoblots from lenses cultured for 5 days (right). Insoluble protein from normal lenses contained α-spectrin at approximately 280 kDa (solid arrow) and 145- and 150-kDa α-spectrin fragments (stippled arrows). Insoluble protein in cultured lenses with A23187 showed decrease in band for α-spectrin at approximately 280 kDa, and increased-bands at 145 and 150 kDa by 1 day. The 150-kDa fragment then decreased and the 145-kDa fragment increased by day 3. SJA6017 and E64 partially prevented theses changes.

kDa. This is a typical pattern resulting from proteolysis by calpain [7,8]. Treatment with SJA6017 or E64 reduced proteolysis of crystallins observed in cataract induced by A23187, while leupeptin did not prevent proteolysis (Fig. 7, upper). Measurement of the α-crystallin band in the gels by image analysis confirmed the effect of inhibitors (Fig. 7, lower). A positive relationship between inhibition of nuclear opacity and inhibition of proteolysis by calpain inhibitors in cultured lenses was observed.

Proteolysis of spectrin and inhibition by cell permeable calpain antagonists have been reported [9]. Insoluble protein from fresh (non-cultured) lens contained intact α-spectrin and lower molecular weight fragments at 145 and 150 kDa; their concentrations were not changed when lenses were cultured in normal medium for 5 days (Fig. 8, left; lanes Fresh versus Normal). Intact α-spectrin in the lens insoluble fraction decreased in A23187-induced cataract, and lower molecular weight fragments at 145 and 150 kDa increased by 1 day (Fig. 8, left; lane A23187). The 150-kDa fragment then decreased and the 145-kDa fragment increased by day 3. This proteolysis increased with culture time. SJA6017 and E64 partially prevented the decrease in intact α-spectrin and 150-kDa fragment, and the increase in the 145-kDa fragment (Fig. 8, left). Leupeptin did not prevent proteolysis of α-spectrin observed in the cataractous lenses. Measurement of each band in the blots by image analysis confirmed the effect of inhibitors (Fig. 8, right). Similar result was observed in soluble protein from cultured lenses (data not shown).

In summary, the studies above report a new, reversible, cell-permeable calpain inhibitor, SJA6017,

which may possess great efficacy against calcium-induced models of rodent cataracts. Results of this experiment suggest future studies to test if SJA6017 is effective in reducing some of pathology associated with cataract in rodent models. The hope is that someday it may be possible to test if SJA6017 is beneficial in preventing some of the pathology in human cataract.

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